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Jerome Anaya
Saint Mary's College

Donald J. Kraft
Saint Mary's College

A Disc Electrophoretic Procedure for Separating Proteins of Marchantia Polymorpha: Esterases.

JEROME ANAYA* and DONALD J. KRAFT**

ABSTRACT — Extracts from Marchantia polymorpha were used in the development of a disc electrophoretic procedure for the separation of proteins. Dialyzed and fresh extracts were found to produce best resolution when electrophoresed in Tris-Glycine buffer (pH 8.3) and 50 millamps current for 45-60 minutes. The esterase enzyme system was used as an indication of separation. Significantly, 5 bands were detected in induced thalli attesting to the sensitivity and application of this method to developmental studies.

Much recent study in the biology of evolutionary, taxonomic and developmental differentiation has turned to the molecular approach. Biochemical methods have proved to be especially important in both zoological and botanical morphogenesis (Leone, 1964; Zeldin and Ward, 1963). In the hepatophyte *Marchantia polymorpha*, various morphological characteristics are exhibited according to environment. Various factors, such as day length, temperature, light (intensity and quality) and humidity, affect the presence or absence of asexual (gemmae) and sexual (gametangiophores) forms of tissue (Wann, 1925; Schappelle, 1936; Scott, 1963).

Earlier work in this area employed starch-gel electrophoresis (Maravolo, Garber and Voth, 1967). The method in this procedure was basically the technique as developed by Ornstein (1964) and Davis (1964). Esterase was chosen as the indication of electrophoretic separation because of its numbers in sexually differentiated *Marchantia* and because of its relatively short assay detection time.

Living plants *Marchantia plants* were collected from Gilmore Creek along the east side of Saint Mary's College campus, Winona, Minnesota. Additional culture plants were obtained from Farmer's Park, also in Winona County.

Plants were sorted for the healthiest thalli. Thalli about 4 cm long were placed on Fiberglass cloth resting on a shallow glass dish, which in turn was placed in an additional tray. Schneider M-5 culture solution (Schneider, 1967) was poured into the outer tray. Four hundred ml was sufficient for supplying nutrients to the plants by capillary action. The culture solution was changed every three days, since the M-5 turns alkaline from plant use (Voth, 1943). Fiberglass proved to be an excellent culture substrate because it did not rot and provided a secure base for rhizoid formation and attachment. Thalli normally attached within two days of planting, as originally observed by Schneider (1967).

Lighting for the plants was provided by a pair of 24″ Sylvania "Gro-Lux" lamps set 18″ above the culture trays. A photoperiod of 12 hours was maintained.

Preparation of protein extracts

Protein extracts were obtained by homogenization, centrifugation and dialysis. Steward and Barber (1964) recommended 1 ml of buffer to 1 gm of tissue as a suitable ratio for homogenization. From preliminary work it was determined that a ratio of 2.5 ml of buffer to 1 gm of tissue was necessary because of the amount of plant material available and the low protein concentration in the plant itself.

Selected tissue was mixed with cold phosphate buffer (pH 6.5) and 5 gm of washed sand or glass beads. The mixture was ground with mortar and pestle for 3-5 minutes for initial maceration. The Biosonik III (Bronwill Scientific Division, Will Scientific, Inc, Rochester, N.Y.) was then used for further solubilizing of protein. Four 30-second treatments with the catenoidal horn operating at 70 per cent efficiency were sufficient for thorough disruption of tissue. It is important that sonication be conducted in an ice bath and that the homogenate be allowed to cool between ultrasonic treatment. These steps are necessary because of the heat generated by ultrasonic energy (6-7 degree rise in temperature per minute of operation at 70 per cent efficiency). Such continued thermal changes can, of course, denature enzymatic proteins.

The homogenate was then strained through glass wool and the solution centrifuged for 30 minutes at 12,000 *g* to remove cellular debris. It is possible to use this supernatant for protein samples on electrophoresis gels. However, the usual procedure was to concentrate the supernatant by ammonium sulfate precipitation at a saturation of 50-60 per cent as suggested by Maravolo *et al.* (1967). This saturation value produced the best results for esterases. The centrifuged pellet obtained from precipitation was resuspended in one-half the original volume of 0.1 M phosphate buffer (pH 6.5).

At this point the resuspension was dialyzed against a 0.1 M phosphate buffer (pH 6.5) for 24 hours. This extract could be stored, but prior to use the pH was adjusted to 8.3. A second method yielding extract for immediate use employed dialysis of the resuspension in a

* JEROME ANAYA is a Sacred Hearts brother and a 1969 graduate in biology at Saint Mary's College, Winona, Minnesota. He is conducting research on the relationship between enzyme activity and development in plants.

** DONALD J. KRAFT, assistant professor, Saint Mary's College, Winona, Minnesota, received his B.S. in chemistry (1959) and his Ph.D. degree in plant physiology (1968) from North Dakota State University. Principal research interests have centered on molecular events surrounding germination and dormancy in plants and isolation and purification of enzymes.

0.1 M Tris-Glycine buffer, pH 8.3 (Steward and Barber, 1964).

Electrophoresis

The extracts were separated according to the method of Davis (1964) and the modifications of Kunske (1967). A 0.1 M Tris-Glycine buffer (pH 8.3) was used in the buffer chambers. A constant current of 50 milliamps was applied for 45-60 minutes or until the tracking dye, bromphenol blue, had migrated to within 5 mm of the end of the gel. Gels were incubated for 1 hour at 25° in a mixture containing 75 mg of Fast Red TRN (Dajac Laboratories, Borden Chemical Co., Philadelphia, Pa.) which was dissolved in 2 ml of 1 – Naphthyl acetate in 50 per cent acetone. Five ml distilled water was added and the entire mixture was agitated with 100 ml of 0.1 M phosphate buffer pH 6.5 (Davenport, 1960).

The effect of various buffers and pH's on migration and separation of esterase is shown in Table I. Several buffer and pH combinations were tried until the Tris-Glycine buffer (pH 8.3) gave satisfactory banding. Buffer systems using pH's below 8.0 produced little or no separation because of the small net charge the molecules exhibited at these pH's. Those systems with pH's from 8.0-8.3 were possibly affected by poor protein samples at the time of experimentation. It is also likley that the buffer combination could have been incompatible with the protein. Additional work is required to substantiate these suppositions.

Gels developed for esterases gave significant reactions unless the protein sample was too old or the buffer system produced no migration. Attempts to stain with Amido Schwartz failed consistently even though gels in the same run gave positive esterase reactions. The reason for this difficulty is not presently known. It could be that the protein sample was too dilute to stain with Amido Schwartz or that diffusion of the protein occurred before staining. The esterase reaction was positive in the mate gels of these instances because the assay was more sensitive and specific than the general protein staining of Amido Schwartz. It is possible also that sample gel composition can lead to separation problems. Gelation time differed with various sucrose percentages in the sample gel. Forty per cent and 80 per cent sucrose ratios normally hardened within 15-20 minutes, as also noted by Davis (1964), whereas the 60 per cent sucrose ratio hardened slowly or not at all. These early test results were utilized as qualitative indicators, so this condition was deemed suitable. However, future studies involving quantitation would need a stronger sample that stays in the gel form. From this standpoint a sample gel with 80 per cent sucrose and a 3:5 sample to gel ratio would be recommended.

Also insignificant from a qualitative standpoint was the age of the extract. Fresh or stored extracts produced no difference in results. The same visible bands were present for both types of extract when assayed from the same gel run.

Densitometric tracings of gels revealed 5 esterase bands. The largest band of the tracings was similar to Maravolo's band h in that it was both the largest band and the leading one. The remaining 3 or 4 bands of the tracings could not be distinguished from Maravolo's bands a-f (1967). Since this work used induced and non-induced thalli but no antheridial discs, the 5 bands present could show a step-by-step increase of enzymes. Where Maravolo *et al.* (1967) found 2 sites in induced and non-induced thalli and additional esterase sites in the antheridia, this method reveals that the induced tissue prior to antheridial formation already contains some of the 6 sites.

TABLE 1 – Buffer systems and their effect on migration and separation of esterase.

Buffer	pH	Migration	Separation
Na_2HPO_4-KH_2PO_4	7.4	minimal	none
Tris-HCl	7.5	minimal	none
Tris-EDTA-Boric acid	7.4	minimal	none
Tris-EDTA-Boric acid	8.0	minimal	none
Tris-EDTA-Boric acid	8.3	none	none
Tris-HCl-Glycine	7.3	none	none
Barbital-HCl	7.3	none	none
Tris-Glycine	8.3	excellent	excellent

These data indicate that separation of *Marchantia* proteins can be accomplished on polyacrylamide gels in a Tris-Glycine buffer (pH 8.3). It is to be noted that the enzyme system may not be directly related to differentiation, although there seems to be a strong correlation. The possibility that other enzyme systems, presently undetected, also may be at work cannot be disregarded. In addition, the substrate used for detection in this study was not the natural substrate. Application of this method seems appropriate for monitoring molecular events, as it can be applied to all other proteins for which there is a specific test. This work is a preliminary development of a procedure which can be applied to further studies of the phosphatases, peroxidases and other enzyme systems of *Marchantia*.

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PLANT PATHOLOGY

Detecting Oak Wilt by False Color Infrared Aerial Photography

R. P. LATHAM,* D. W. FRENCH,** MERLE P. MEYER***

ABSTRACT — An aerial survey using false-color infrared film at scales of 1:9,600 and 1:12,000 located 93.7% of the oak wilt infection centers in the 1,500-acre study area. The ground survey of the same area also located 93.7% of the infection centers but half of those found by this method were difficult to relocate because they were not correctly mapped. Whereas aerial survey usually missed only trees of small diameter, the ground survey missed trees as large as eight inches in diameter.

Oak wilt, caused by *Ceratocystis fagacearum* (Bretz) Hunt, is a long-established and widely distributed tree disease in the Upper Midwest. It causes widespread depreciation of property values, particularly in suburban areas where red oaks are often the primary shade tree species. Red oaks are killed rapidly by this fungus, dying within a few months after symptoms become evident. The fungus also can kill white oaks, although these species may survive for a year or longer after symptoms appear.

Present control methods require one or more complete ground surveys each summer so that all of the infected red oaks can be detected and removed before spores are produced the following spring to cause new infections. The spores are produced on mycelial mats which develop between the wood and bark. Although a detection and removal program does not eradicate the disease, it does reduce annual losses and helps to provide time for introduction of other tree species as replacements for the oaks. Complete ground coverage is, of course, slow and expensive. If ground crews are not well versed in the use of maps, given locations are often erroneous and the diseased trees are not eradicated. Also, since the disease first becomes apparent in the very top of the tree, observation from the ground may miss trees which are just beginning to wilt.

Comparison of survey methods

A portion of the Village of North Oaks, a suburb of St. Paul, was selected for comparison of aerial survey with inspection from the ground as a means of detecting infected trees. This community is well suited to such a comparison because of the large number of oaks (approximately 75,000) and the incidence of oak wilt, which has ranged from 236 to 284 infected red oaks annually since 1963.

Stereoscopic aerial protographs were taken, using Kodak Ektachrome Infrared Aero Film, Type 8443, a Zeiss RMK A 15/23 9x9-inch format camera, and a Wratten 12 filter, at scales of 1:9,600 (1″ = 800′) and 1:12,000 (1″ = 1,000′). Two survey flights were made over the area by Mark Hurd Aerial Surveys during the first week of August, 1967. The developed film strips were viewed over a light table with a scanning stereoscope, and the suspected diseased trees were marked on acetate overlays placed over the color transparencies. Two photo interpreters, without prior ground experience in the study area, each examined both the 1:9,600 and the 1:12,000 scale photography for a total of four interpretations of the community. Once the annotation of the acetate overlays was complete, a black and white internegative was made from each color exposure so that regular black and white contact prints could be produced. A photo mosaic was then prepared from black and white contact prints and the locations of suspected trees were transferred from the acetate overlays to the mosaic for use in checking the trees from the ground.

The essential part of the system is the film and filter combination. This false-color, reversal type film, when used with a minus-blue filter, is sensitive to the green, red, and near-infrared portions of the spectrum. Healthy vegetation, having a high reflectivity in the infrared wave

The authors, all at the University of Minnesota, are respectively: * Research Fellow, School of Forestry; ** Professor, Department of Plant Pathology; *** Professor, School of Forestry.

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